STUDY OF RELATIONSHIP BETWEEN SUSCEPTIBILITY TO VARIOUS CANCERS AND ARYL HYDROCARBON HYDORXYLASE (AHH) INDUCIBILITY

PROGRESS REPORT

April 21, 1975

Period covered: November 1, 1974 - April 30, 1975

Summary:

Our essential aim is to test the published results of Shaw and the Kellerman's that an individuals AHH inducibility is strongly related to his lung cancer risk.

The core problem of this study still remains to be solved as of this date, viz. the establishment of a repeatable test. As we stated in our previous report the chemistry part of the test has been satisfactorily established and we have made further improvements in it recently. But the variability of AHH inducibility in peripheral blood lymphocytes between split-samples of bloods run in parallel is much too great for comfort, and the day-to-day variation is considerably worse: this is true whether the analysis is done in Dr. Kouri's laboratory or in our own laboratory.

Dr. Kouri continues to work on the AHH assay of peripheral blood lymphocytes, but, because of these problems, we have shifted recently from these cells to cultured cell lines. This change has given us a continuing source of "identical" cells to work with and, although the AHH activity is no greater than stimulated lymphocytes, we have managed to produce much less variable results and to identify the main sources of variation in results.

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We should be able during the next few months to establish a straightforward, easily duplicated and repeatable test for AHH inducibility in fibroblast cell lines. Although this test will clearly be considerably more difficult to use than a test based on peripheral blood lymphocytes, it should at a minimum permit testing of the original observations of Kellerman, Shaw and Luyten-Kellerman on the relationship of AHH and lung cancer. It should also provide a test system against which more convenient systems can be judged. If no more convenient systems are found it will be usable for the other epidemiologic projects we planned.

Arrangements for collecting blood from cases and controls remain operative and there will be no trouble supplying Dr. Kouri with samples again once his test system is firmly established. If we need to use a fibroblast test, new arrangements will have to be made to collect specimens - we envisage no real problems in doing this.

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Detailed Progress

(a) Spectrophotofluorometer (SPF) assay for 3-hydroxybenzo(a)pyrene (3-HOBP)

As stated in our last report this aspect of the assay was essentially workable once we began to employ nitrogen purges of SPF cuvettes, etc. However, since we began to work with cell lines and our results became more reproducible we found that this part of the test system was itself producing significant variation due to traces of the acetone-hexane mixture being transferred into

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the SPF cuvette on the sides of the pipette used for the transfer of the final NaOH layer: we have corrected this problem by changing the SPF layout to accommodate the test-tube with the acetone-hexane plus NaOH layer and eliminating the necessity for a transfer to the special small cuvette.

(b) Experience with complete peripheral blood lymphocyte test system

Table 1 shows some results from samples we sent to Dr. Kouri. (A considerable number of samples produced no results due to problems with air shipment resulting in non-viability of cells received in Bethesda - these problems have now been sorted out.) It is clear from this table that the test system used has major problems.

Generally repeatability of split-samples (testing all done blind by Dr. Kouri with coded samples) is not too bad but every fourth or fifth subsample gives a wild result. For example, a blood sample from O.D. split four ways gave AHH inducibility of 4.7, 4.3, 4.1 and 1.6. The first three results are probably acceptable as repeats, but clearly the 1.6 value is not. Repeatability from one week to the next is considerably worse. The first split-sample from A.E. averaged out at about 3 whereas the second averaged out at about 11.

Results we obtained in doing the test in our own laboratory were if anything even more variable than Dr. Kouri's.

A number of possible reasons for this failure to successfully establish the test system can be given. In particular, one may

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ask whether the PHA activation of the lymphocytes is just too variable.

Dr. Kouri is continuing work to try to sort out these difficulties. We decided, however, that we would retreat to what we hope will be a more easily standardized test using cell lines.

(c) Assaying AHH inducibility in cultured cell lines

Lymphocytes in vivo probably do not use their AHH enzyme system to any great extent and this may account for the extremely low amounts of AHH detected in 3MC induced lymphocytes in vitro (the above test system). This is certainly one of the problems with the lymphocyte test.

We decided therefore to measure AHH activity both in the most accessible cell line system, viz. fibroblasts, in order to obtain an epidemiologically usable test, and in liver and other cell lines in the hope of finding a line with high levels of AHH activity. The latter, if obtainable, should enable us to sort out more easily the best method of inducing AHH activity, etc.

The Chang liver cell line unfortunately does not produce high AHH activity as we had hoped, but tests on it did show up some of the problems and variables that need to be sorted out even in a "uniform cell population" AHH assay system.

Results from our last two experiments with "Chang" liver cells were as follows:

Experiment #15 - Chang cells from culture flasks were removed by trypsin, pooled, counted and distributed in amounts varying

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from 0.4×10^6 - 10.0×10^6 cells into 12 new flasks as shown in Table 2. Culture media was added to obtain a fixed volume of 10ml; 100λ 3MC was then added in the standard lymphocyte assay manner and the cells induced for 24 hours. The cells were then trypsinized, counted and assayed for AHH activity in the standard manner using BP for 45 minutes. The AHH activity (in SPF units) is given in the table (these results multiplied by 24 give a rough estimate of activity in picomoles).

Experiment #16 - Same procedure as for Experiment #15 done 1 week later. Results given in Table 3.

The results of both experiments are plotted in Figures 1 and 2. Other experiments on Chang cells at an initial number of 0.8×10^6 per flask have shown that the BP for zero minutes baseline control gives readings around 0.18 (in SPF units). Using this as the zero cells intercept (an assumption now being tested by running zero time controls with other cell numbers) we see that we may tentatively draw the conclusion that induction is probably linear up to 2×10^6 cells per flask and reproducibility week to week is probably achievable with this system. One problem is removing all cells from the flasks after induction (we are currently experimenting with Petri dished in a CO_2 atmosphere). Another is measuring substrate mass (e.g. should one use number of cells, quantity of protein or, as in certain mouse experiments, the weight of tissue? We suspect that conventional cell counting introduces a not insignificant amount of variability.

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Experiments with a fibroblast line ('1609') show a number of differences from these results with Chang cells. The peak SPF reading is obtained at a cell number of 1.2×10^6 cells and rather than simply flattening off when the cell numbers exceed this it drops sharply off. The peak SPF reading is however more than twice as high as Chang cells.

We are therefore concentrating on the '1609' cell line at present (and growing up other fibroblast lines for further work). On the basis of our work to date we presume that the "best" test system will be:

(1) Cultivation of cells in flasks.

(2) Removal with trypsin 72 hours before 3MC induction into Petri dishes

(3) Petri dish induction with 100λ 3MC at a cell number of 10^6/dish.

(4) Removal with trypsin and tube incubation with 100λ BP for 45 minutes.

Possible modifications requiring testing are:

(a) Mechanical handling of cells: does trypsin damage the cells? Would we do better at step (4) with a rubber policeman?

(b) Cell substrate measurement: would we get more repeatable results if we measured protein mass instead of number of cells.

(c) Is 3MC the 'best' inducer? (There is some evidence that 3MC does not induce monocytes.) How much 3MC should we use?

(d) Does the amount of BP added have any effect?

When these questions are answered there will be a number of possible ways to go, chosing priorities will depend on the progress other laboratories have made in the intervening period.

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(i) Apply the knowledge of optimum system for fibroblasts to other human tissues (including PHA stimulated peripheral blood lymphocytes) which do not require establishing cell lines.

(ii) Test a limited number of lung cancer cases and controls using fibroblast cell lines. This will be a lengthy procedure.

(iii) Test other cell line types for AHH inducibility to gain information on metabolism of hydrocarbons in different tissues.

If the stimulated lymphocyte test is established by Dr. Kouri (or any others) we will, of course, pursue as first priority our original plan of testing cases of cancer at various sites and controls.

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Table 1
Results of Specimens Sent to Dr. Kouri

Name	Date	AHH#	Constit.	Induced	I/C
A.E.	10/10	116	.96	2.2	2.3
	10/10	117	1.0	3.05	3.1
	10/10	119	.51	2.0	3.9
	10/10	121	.44	1.64	3.7
	10/24	153	1.0	14.0	14.0
	10/24	154	1.91	17.2	9.0
	10/24	156	1.35	9.7	7.2
	10/24	158	.84	10.0	11.9
	1/ 8	228	.022	.44	20.0
	1/ 8	229	.047	.34	7.2
	1/ 8	232	-----	-----	no growth
	1/ 8	233	.022	.18	8.2
A.A.	12/11	206	.094	.42	4.5
	12/11	207	.1	.41	4.1
	12/11	208	.099	.58	5.9
	12/11	209	.099	.47	4.7
A.R.	10/ 2	74	6.9	23.3	3.4
	10/ 2	75	2.5	10.0	4.0
	10/ 2	88	2.25	5.4	2.4
	10/ 2	90	1.42	4.2	3.0
	10/ 9	105	1.2	3.4	2.8
	10/ 9	108	0.60	2.4	4.0
	10/ 9	112	1.10	3.4	3.1
	10/ 9	113	1.25	4.0	3.2
A.J.	10/ 9	103	.56	3.15	5.6
	10/ 9	107	.62	2.72	4.4
	10/ 9	109	.43	2.78	6.5
	10/ 9	111	.885	3.9	4.4
B.J.	1/ 8	226	0.35	1.32	3.8
	1/ 8	227	----	-----	no growth
	1/ 8	230	0.74	1.85	2.5
	1/ 8	231	0.35	1.80	5.1
C.J.	8/21	11	1.68	4.52	2.7
	10/17	134	-----	-----	no growth
	10/17	137	-----	-----	no growth
	10/17	139	-----	-----	no growth
	10/17	141	-----	-----	no growth
	12/11	202	.075	.134	1.8
	12/11	203	.032	.136	4.3
	12/11	204	.065	.250	3.9
	12/11	205	.065	.220	3.4

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Name	Date	AHH#	Constit.	Induced	I/C
Chu. M.	10/ 2	77	3.5	7.0	2.0
	10/ 2	78	0.77	1.32	1.7
	10/ 2	89	0.52	.78	1.5
	10/ 2	91	0.61	1.96	3.2
	10/ 9	102	0.5	1.9	3.8
	10/ 9	104	0.35	1.0	2.9
	10/ 9	106	0.52	1.71	3.3
	10/ 9	110	-----	-----	no growth
	11/ 7	188	-----	-----	no growth
	11/ 7	189	-----	-----	no growth
	11/ 7	192	.57	3.55	6.2
	11/ 7	193	.56	3.1	5.5
Cof. G.	10/ 3	92	-----	-----	no growth
	10/ 3	93	-----	-----	no growth
	10/ 3	95	-----	-----	no growth
	10/ 3	99	-----	-----	no growth
Cof. M.	10/30	162	3.6	8.3	2.3
	10/30	163	3.9	8.9	2.3
	10/30	165	2.55	5.8	2.3
	10/30	167	2.96	7.33	2.5
	2/12	214	.11	.37	3.4
	2/12	215	.16	.495	3.1
	2/12	216	.09	.356	4.0
	2/12	217	.122	.71	5.8
D.A.	10/23	144	.39	1.18	3.0
	10/23	147	1.15	3.8	3.3
	10/23	151	1.4	4.9	3.5
	1/ 9	234	.078	.40	5.1
	1/ 9	236	.040	.13	3.3
	1/ 9	238	.06	.35	5.8
	1/ 9	240	.05	.183	3.7
H.M.	10/ 3	94	-----	-----	no growth
	10/ 3	96	-----	-----	no growth
	10/ 3	97	-----	-----	no growth
	10/ 3	98	-----	-----	no growth
	10/24	152	.88	4.7	5.3
	10/24	155	1.16	6.3	5.4
	10/24	157	1.31	5.1	3.9
	10/24	159	.76	4.9	6.4
	11/ 7	190	-----	-----	no growth
	11/ 7	191	-----	-----	no growth
	11/ 7	194	.95	5.6	5.9
	11/ 7	195	1.2	5.2	4.3

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Name	Date	AHH	Constit.	Induced	I/C
H.R.	10/10	114	-----	-----	no growth
	10/10	115	.42	2.5	6.0
	10/10	118	.58	2.5	4.3
	10/10	120	.45	1.66	3.7
M.L.	10/23	145	1.22	6.9	5.7
	10/23	146	.54	4.6	8.5
	10/23	148	.42	6.3	15.0
	10/23	150	.53	4.0	7.5
N.M.	1/ 9	235	.071	.31	4.4
	1/ 9	237	.073	.30	4.1
	1/ 9	239	.089	.498	5.6
	1/ 9	241	.094	.32	3.4
O.D.	10/31	170	.78	1.24	1.6
	10/31	172	.54	2.56	4.7
	10/31	174	.88	3.6	4.1
	10/31	176	.88	3.8	4.3
P.M.	8/21	8	3.15	10.91	3.5
	10/ 2	81	3.0	10.0	3.3
	10/ 2	82	5.2	10.7	2.1
	10/ 2	84	2.9	7.9	2.7
	10/ 2	86	4.5	9.0	2.0
S.M.	10/31	171	.44	2.75	6.3
	10/31	173	.42	2.65	6.3
	10/31	175	.64	1.15	1.8
	10/31	177	.51	2.30	4.5
S.A.	8/21	9	1.88	2.67	1.4
T.R.	10/30	164	1.64	4.6	2.8
	10/30	166	2.25	6.75	3.0
	10/30	168	2.5	7.7	3.1
	10/30	169	2.0	5.15	2.6
	12/12	210	.093	.41	4.4
	12/12	211	.11	.75	6.8
	12/12	212	.14	.5	3.6
	12/12	213	.16	.55	3.4

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Table 2
Experiment #15
Chang Cells induced with 100λ MC

Flask #	# cells (10^6) at 0 hrs.	# cells (10^6) at 24 hrs.	SPF readings
1	.4	.465	.274
2	.6	.855	.344
3	.8	.855	.329
4	1.0	.810	.407
5	1.2	1.73	.410
7	1.6	2.04	.514
8	1.8	1.80	.529
9	2.0	2.43	.380
10	4.0	3.92	.459
11	8.0	5.55	.555
12	10.0	4.19	.578

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Table 3
Experiment #16
Results with Chang Cells induced with 100λ MC

Flask #	# cells (10^6) at 0 hrs.	# cells (10^6) at 24 hrs.	SPF reading
9	.4	.289	.238
10	.6	.525	.307
12	.8	.705	.341
13	1.0	1.26	.337
14	1.2	1.43	.391
15	1.4	1.39	.474
16	1.6	1.72	.542

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KEUFFEL & ESSER CO. MADE IN U.S.A.
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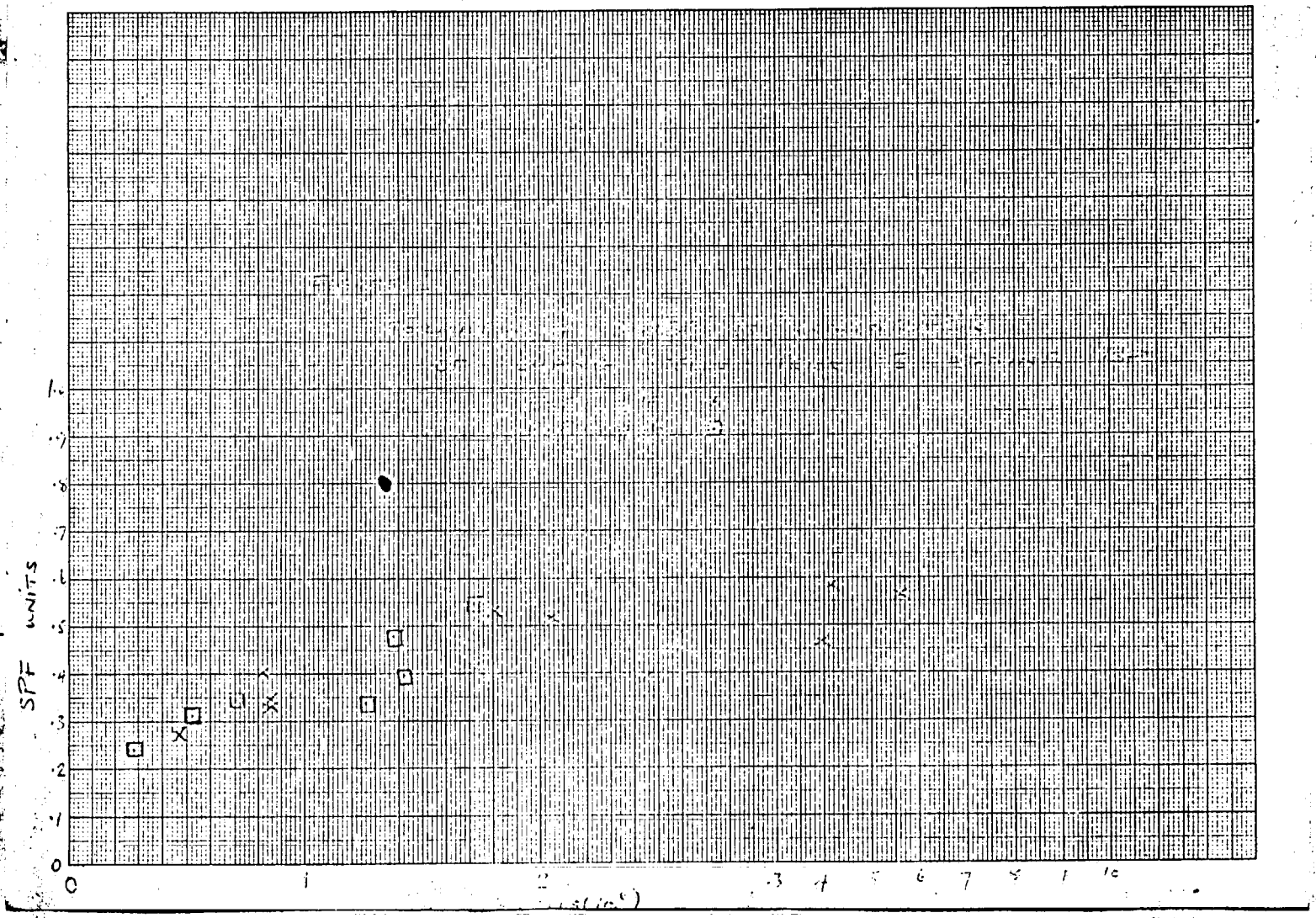

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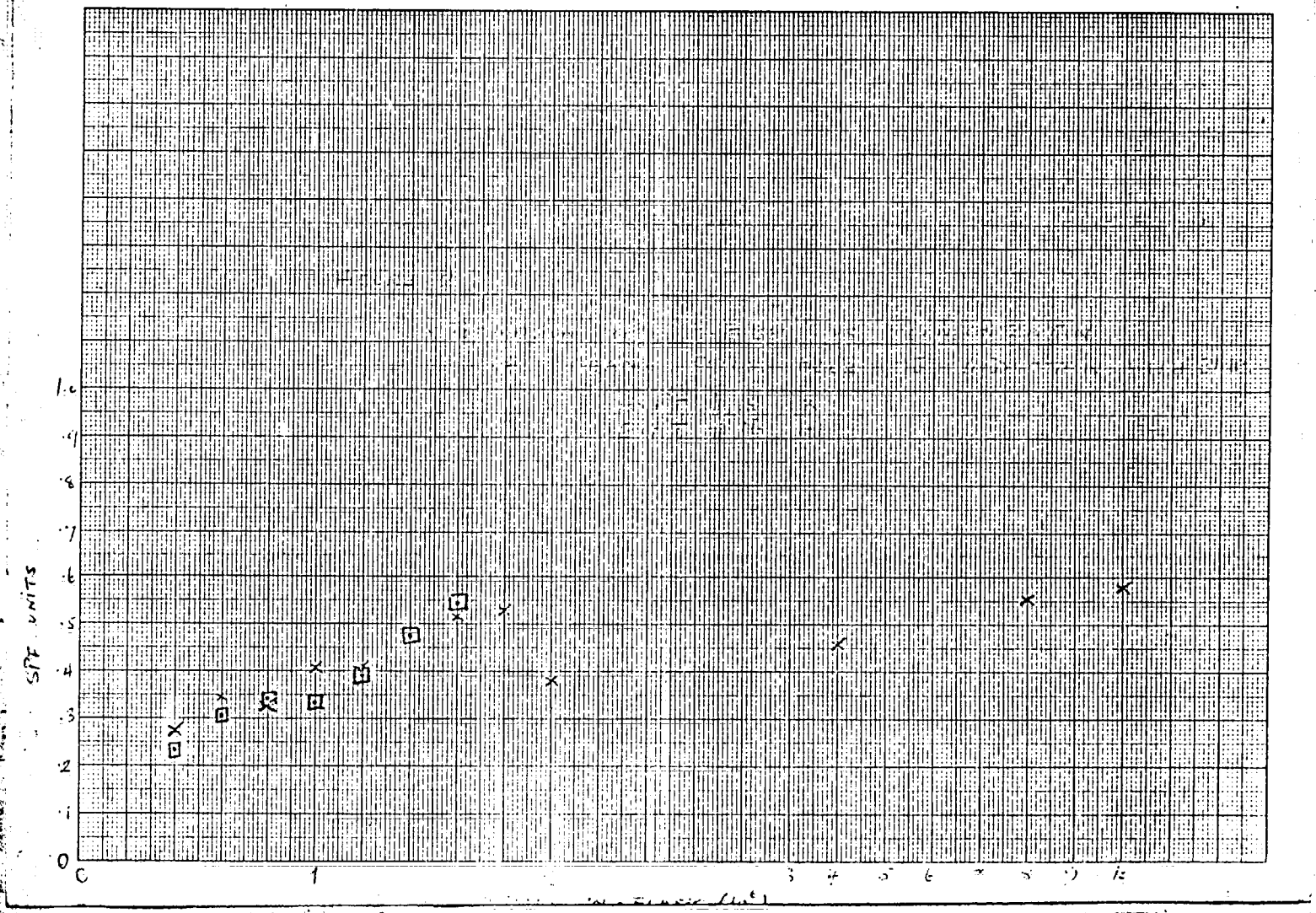

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